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Application of: **Jon A. Wolff, Paul M. Slattum, James E. Hagstrom, Vladimir G. Budker**

Serial No.: **09/631,152**

Examiner: **Daniel M. Sullivan**

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Group Art Unit: **1636**

For: **Gene Expression With Covalently Modified Polynucleotides**

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
PO Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

I, James E. Hagstrom, hereby declare as follows:

1. I am an inventor of the captioned application.
2. I submit with this Declaration and Response further experimental material (attached) illustrating: delivery of polynucleotides labeled with differently sized tags within the expression cassette which were delivered and expressed. The experiments were performed according to the methods provided in the Specification.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

J. E. Hagstrom 9/17/04
James E. Hagstrom Date

DECLARATION EXAMPLES

Example 1:

Goal: to covalently attach nuclear targeting peptides to the pEYFP-Nuc plasmid DNA at random positions in order to evaluate the effect of this nuclear localization signal (NLS) on enhanced marker gene expression.

Methods:

1. The peptides:
   The short SV40 large T-antigen NLS peptide was used as a targeting peptide, and a His(5) oligo-histidine peptide was used as a negative control. Both peptides carried a cystein on the N-terminus, providing a specific attachment site for sulfhydryl-based chemistry:

SH-CGYGPKKKRKVGG-OH	SV40 NLS (the minimal NLS signal underlined)
SH-CHHHHH-OH	His(5)

   The peptides were attached to our sulfhydryl-specific DNA alkylating nitrogen-mustard R-chloride reagent to create NLS-LabelIT and His(5)-LabelIT.

2. Labeling the DNA:
   The 4.8 kb plasmid pEYFP-Nuc (Clontech, CA) encodes the enhanced yellow-green variant of the Aequorea victoria green fluorescent protein (GFP) gene driven by the strong CMV promoter. The expressed GFP has three tandem copies of the SV 40 large T-antigen NLS fused at its C-terminus, which causes it to localize to the nucleus, and strongly to the nucleolus of cells, greatly enhancing detection of the protein even at low levels of expression. This marker gene thus provides a sensitive assay to monitor gene expression in microinjected cells early after the onset of transcription. The pEYFP-Nuc pDNA was labeled with either no peptide (mock-treated control), with the NLS-LabelIT peptide or with the His(5)-LabelIT peptide at 1:0.1 DNA to peptide weight ratio, for 1 hour at 37°C. The three DNA samples were purified by ethanol precipitation and were dissolved in water.

3. Microinjections:
   HeLa cells were plated on CellLocate glass cover slips (Eppendorf) containing a grid of 55-μm squares, in 35 mm dishes. 24 hours later individual cells at least 100 μm apart were microinjected using an Eppendorf 5246 Transjector and 5171 Micromanipulator. All injections were done with 0.5 psi pressure for 0.3 sec, targeting the cytoplasmic compartment of the cells. To mark the injected cells and to verify the injected compartment in undivided cells, all samples contained 0.4 mg/ml 500 kDa LissamineRhodamine-Dextran in a 5% isotonic glucose solution buffered with 20 mM HEPES pH 7.4. In undivided cells dextran of this size remains excluded from the nuclear compartment, unless the injection damaged the nuclear envelope. Cells with compromised nuclei were excluded from the analysis. (Divided cells could be recognized by detecting 2 sister-cells in close vicinity containing the fluorescent dextran, while undivided cells appeared as individual cells far from any neighboring injected cell.) All microinjection samples contained the pDNA at a 5 ng/μl final

concentration. The microinjected cultures were incubated for 8 hrs post-injection, followed by 2 PBS washes and fixation in 4% formaldehyde. Cover slips were mounted in Gel/Mount (BioMeda) and the cells were observed under a Zeiss LSM 510 confocal laser scanning microscope. EYFP-expressing cells were counted both in the "undivided" and the "divided" categories. When divided cells expressed, they all showed marker gene expression in both sister cells, thus each pair was counted as "one" originally injected parent cell.

Results:

The following table shows the percentage of EYFP-expressing cells 8 hours after microinjections into the cytoplasm of HeLa cells using four different DNA samples:

Description of samples	Percent of EYFP-positive cells in		
	undivided cells	divided cells	total number of injected cells
pEYFP-Nuc "mock-treated"	3	50	17
pEYFP-Nuc NLS-LabelIT labeled	10.3	28.6	12.5
pEYFP-Nuc His(5)-LabelIT labeled	1.5	20	5
pEYFP-Nuc untreated	13.7	27.3	15.5

Conclusion:

The peptide-modified pDNA samples were able to express the marker gene, albeit at reduced rate. The DNA:peptide ratio used for the alkylation step is estimated to result in the attachment of 0.5-1.0 peptide per 100 basepair. Considering the < 1 kb size of the EYFP gene, this translates into 5-10 peptides attached within the coding region. This had a mild inhibitory effect on transcription.

The attachment of the NLS peptide to the pDNA increased expression levels in undivided cells compared to the negative control His(5) peptide, but it did not yield higher expression levels than the untreated or "mock-treated" pDNA. This effect could not be observed in cells that went through mitosis during the 8 hours of incubation. Apparently, in those cells all the four DNA samples had a similar chance to enter the nuclear compartment. In summary, this approach was not suitable to increase overall expression levels by the enhancement of the nuclear entry of pDNA.

Example 2:

Goal: to indirectly attach nuclear targeting peptides to the luciferase expressing pMIR048 plasmid DNA at random positions in order to quantitate the effect of the NLS on enhanced marker gene expression.

Methods:

1. The peptides:
   The long version of the SV40 large T-antigen NLS (LSV40-NLS):
   SH-CKKKSSSDDEATADSQHSTPPKKKRKVEDPKDFPSELLS-OH

   The nucleoplasmin bipartite NLS (NP-NLS):
   SH-CKKAVKRPAATKKAGQAKKKKL-OH

   Both peptides carried a cystein on the N-terminus, providing a specific attachment site for sulfhydryl-based cross-linking chemistry.

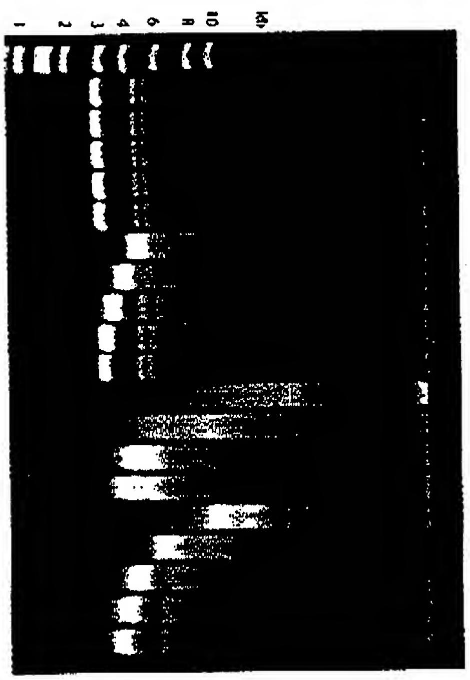
2. Indirect attachment of the peptides to pDNA:
   - The targeting peptides were covalently conjugated to streptavidin (SA) using primary amine groups on the SA, the sulfhydryl groups on the peptides and the heterobifunctional Sulfo-SMCC (Pierce) cross-linker. The standard Pierce protocol was followed. Based on SDS-PAGE electrophoresis there was an average 2 peptides attached per SA subunit, which means approximately 8 targeting signals per tetramer.
   - The pDNA was biotinylated using Biotin-LabelIT at 5 different DNA:reagent weight ratios: 1:0.1, 1:0.05, 1:0.02, 1:0.005 and 1:0 (mock-treated). Reactions were incubated for 1 hour at 37°C. The DNA was purified by two ethanol precipitations and finally dissolved in water. These labeling ratios were expected to result in the attachment of 1, 0.5, 0.2, 0.1 and zero biotin per 100 basepair, respectively. The actual biotin content of the DNA samples was not determined.
   - The biotinylated pDNA samples were either used alone, or were complexed with a large molar excess of plain (unconjugated) SA, NP-NLS-SA conjugate or LSV40-NLS-SA conjugate. An untreated pMIR048 pDNA sample was also used as a control for each condition. The formation of the biotin-DNA + SA complexes were verified by agarose gel electrophoresis (Figure 1).

3. Transfection of HeLa cells:
   HeLa cells were plated into 12-well tissue culture dishes 24 hours prior to transfection. The freshly prepared DNA or DNA+SA samples were complexed with TransIT-LT1 transfection reagent (Mirus) and aliquots containing 1 μg DNA were added to each well. The wells contained 500 μl serum free OptiMem medium. Three hours later the medium was changed for DMEM supplemented with 10% FBS. 20 hours later the cultures were washed with PBS twice and the cells were lysed in 200 μl/well LUX buffer. Luciferase activity was determined in a 5 μl aliquot of each lysate. Enzyme activities were expressed in relative light units (RLU; Figure 2).

Results:

- Indirect attachment of NLS peptides to the pDNA:
When mock-treated, biotinylated and untreated pMIR048 pDNA samples were complexed with an excess of plain SA or peptide-conjugated SA for the transfection, an aliquot containing 400 μg DNA was removed prior to the addition of the TransIT-LT1 reagent. These DNA samples were analyzed on a 0.5% agarose gel. As apparent on Figure 1, the mobility of the biotinylated DNA samples containing SA of any kind was altered due to the

enlarged size of the complexes. The extent of the gel-shift was proportional to the biotinylation level of the DNA, suggesting that the indirect attachment of SA to the pDNA was successful. The highly positively charged (10+) NP-NLS peptide caused more intense gel retardation of the complexes due to partially neutralizing the negative charge of the DNA. This effect is less pronounced with the LSV40-NLS-SA conjugates, since the net charge of that peptide is only 1+. From the gel image it is also apparent that the biotinylation step did not alter the conformation and the mobility of the pMIR048 pDNA at any ratios (Lanes 2-6).



1. Marker
2. 1:0.1 Biotin-DNA alone
3. 1:0.05 Biotin-DNA alone
4. 1:0.02 Biotin-DNA alone
5. 1:0.005 Biotin-DNA alone
6. 1:0 Mock-treated DNA alone
7. 1:0.1 Biotin-DNA + Plain SA
8. 1:0.05 Biotin-DNA + Plain SA
9. 1:0.02 Biotin-DNA + Plain SA
10. 1:0.005 Biotin-DNA + Plain SA
11. 1:0 Mock-treated DNA + Plain SA
12. 1:0.1 Biotin-DNA + NP-NLS-SA
13. 1:0.05 Biotin-DNA + NP-NLS-SA
14. 1:0.02 Biotin DNA + NP-NLS-SA
15. 1:0.005 Biotin-DNA + NP-NLS-SA
16. 1:0.1 Biotin-DNA + LSV40-NLS-SA
17. 1:0.05 Biotin-DNA + LSV40-NLS-SA
18. 1:0.02 Biotin-DNA + LSV40-NLS-SA
19. 1:0.005 Biotin-DNA + LSV40-NLS-SA
20. 1:0 Mock-treated DNA + LSV40-NLS-SA

Figure 1. Mobility of the biotinylated or mock-treated pMIR048 samples in a 0.5% agarose gel with or without added streptavidin.

- Intensity of the luciferase marker gene expression:

As shown in the graph of Figure 2, the luciferase activity of the untreated pMIR048, the Mock-treated control and the biotin-DNA modified at the lowest 1:0.005 ratio were essentially identical when used without added SA. The addition of any kind of SA to the biotinylated DNA, even at this lowest level, resulted in the enzyme activity to drop to 60-80% of the controls. The biotinylated DNA sample modified at 1:0.02 ratio yielded only half as much (50%) luciferase activity as the control, and this was further reduced by the addition of SA to a mere 20% activity of the control. The higher modification levels resulted in even more pronounced reduction of transcriptional activity: Also, the more biotin was attached to the DNA, the more dramatic effect the addition of the SA had. When using the most heavily modified DNA (1:0.1 ratio, resulting in the attachment of an estimated 1 biotin per 100 bp), the addition of SA caused a 15-fold drop in luciferase expression, compared to only 4.2-fold drop in the case of the DNA modified at 1:0.05, and only 2.8-fold decrease for the 1:0.02 ratio. Samples complexed with the NLS-peptide containing SA conjugates yielded slightly higher enzyme activities than the complexes containing plain SA. However, this effect could

be observed in the un-biotinylated control samples, as well, where only electrostatic interactions could take place between the DNA and the NLS-SA conjugates. This suggests that the effect is not the consequence of the indirect, stable, attachment of the targeting peptides to the DNA. It may have an effect on the complex formation with the TransitLT1 transfection reagent, increasing the efficiency of transfection.

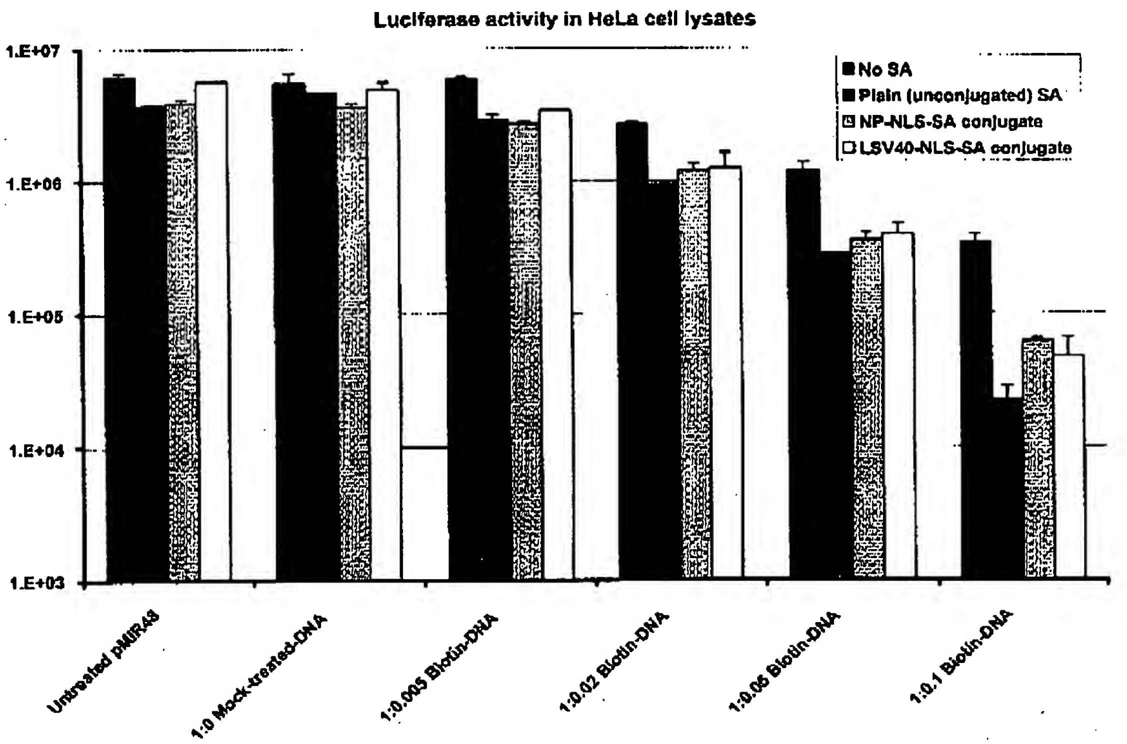


Figure 2. The effect of the indirect attachment of nuclear targeting signals to pDNA on the expression of the luciferase marker gene. pMIR048 pDNA was labeled with various amounts of biotin followed by complex formation with unconjugated or NLS peptide-conjugated streptavidin (SA). The samples were introduced into HeLa cells by LT1-mediated transfection. Luciferase activity was assayed in the cell lysates 20 hours post-transfection.

Conclusion:

The biotin-modified pDNA samples were able to express the marker gene, albeit at slightly reduced rate. The reduction in transcription efficiency was proportional to the modification level. At the highest level of biotinylation this meant a roughly 20-fold drop, while at the lowest level luciferase activity was indiscernible from the mock-treated and untreated samples, as long as the DNA was not complexed to SA. The addition of SA caused further reduction in the transcriptional activity of the modified DNA samples, and this effect was also proportional to the level of modification. This suggests that the size of the adduct on the

DNA makes a difference for transcription inhibition: the attached biotin is tolerated a lot better than an attached > 60 kDa globular protein.

The attachment of the NLS peptide to the pDNA by the indirect SA-biotin bond increased expression levels slightly when compared to the activity of the same pDNA complexed to plain SA. However, this effect was independent of the biotinylation state of the DNA and is thought to be caused by electrostatic interactions between the DNA and the positively charged NLS peptides, possibly modifying the efficiency of transfection rather than the efficiency of nuclear entry. The overall expression levels always lagged behind the expression from the same uncomplexed DNA, and even further behind untreated DNA. Thus, this approach was not suitable to increase overall expression levels by the enhancement of the nuclear entry of pDNA.